SPECIAL SECTION ON FEED ADDITIVES AND CONTAMINANTS

Factors Affecting Accuracy and Time Requirements of a Glucose Oxidase–Peroxidase Assay for Determination of Glucose

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Accurate and rapid assays for glucose are desirable for analysis of glucose and starch in food and feedstuffs. An established colorimetric glucose oxidase–peroxidase method for glucose was modified to reduce analysis time and evaluated for factors that affected accuracy. Time required to perform the assay was reduced by approximately 40% by decreasing incubation time and removing steps that do not affect absorbance. Although linear regressions of absorbance and glucose concentrations of standard solutions exceeded R^2 of 0.9997, evaluation of sum of squared residuals, root mean squared error, and significance of the quadratic term indicated that the curves were approximately quadratic in form. Inadequate equilibration of glucose anomers did not appear to be the issue. Historic data suggest that the standard curve is inherently nonlinear. Quadratic curves predicted standard solution glucose concentrations more accurately than did linear forms; overestimations at the midpoint of the curve averaged 0.04, 0.48, and 0.92% for quadratic and linear equations calculated from 5 standard solutions and a linear equation calculated from the 0 and most concentrated standard solution, respectively. A hydrophilic antioxidant at levels no greater than 10 μmol ascorbic acid/0.10 g air-dried sample did not affect absorbance values.

Enzymatic–colorimetric analyses using glucose oxidase and peroxidase (GOPOD) are commonly used for detection of glucose in methods for free glucose (1), starch (2, 3), and resistant starch (4). The assays are both specific and sensitive for the detection of glucose. There are many permutations of GOPOD assays that vary in composition of the GOPOD reagent, incubation times, ratios of sample to reagent, toxicity of reagents, and other elements. The GOPOD method for glucose described by Karkalas (5) avoids the use of potentially carcinogenic reagents such as *o*-dianisidine, and it gives very repeatable within-assay absorbance values. However, increased sample throughput and more economical use of laboratory resources could be achieved through modifications to the assay to reduce the time required for incubation and sample handling. Introduction of modifications warrants the reevaluation of assay performance in terms of its accuracy in predicting glucose concentration and the effect of potentially interfering substances.

The purpose of the present study was to investigate modifications to the Karkalas (5) GOPOD assay for glucose that would reduce the time required for analysis, and to evaluate factors that affect the accuracy of prediction of glucose in the modified assays.

Experimental

Design

The GOPOD method for glucose analysis described by Karkalas (5) was evaluated in a single laboratory with work performed by one technician. Elements evaluated were the effect of temperature and length of incubation, ratio of sample solution to GOPOD reagent, effect of vortexing samples before incubation, effect of cooling samples in the dark post-incubation, effect of time delay between the end of incubation and reading sample absorbance, linearity of absorbance response, time from preparation of standard solutions to analysis and reading, effect of GOPOD reagent type on linearity of response, and application of standard curves based on 5 versus 2 independently prepared standard solutions. Four to 7 standard solutions were analyzed in triplicate for each treatment within each analysis run. Additionally, the interference of a hydrophilic antioxidant (ascorbic acid) on the detection of glucose carried through a starch analysis procedure was evaluated. Each treatment was evaluated in 2 separate runs, thus giving 2 independent results per assay permutation. All possible combinations of factors were not evaluated for each incubation and sample:reagent

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Table 1. Effect of delayed reading on sample absorbance values and percentage change in absorbance relative to no time delay[a]

		Time delay to reading, min				
Incubation condition	Glucose, μg/mL	0	15	30	45	60
Standard solution:GOPODk reagent (0.5:2.5)		Absorbance at 505 nm				
35°C for 45 min	0	0.000	0.001	0.001	0.002	0.003
	40	0.239	0.238	0.237	0.234	0.236
	60	0.359	0.357	0.354	0.352	0.351
	100	0.594	0.586	0.582	0.579	0.579
		(Absorbance/0 time delay absorbance) × 100				
	40		99.7%	99.1%	98.1%	98.8%
	60		99.2%	98.6%	97.9%	97.8%
	100		98.7%	98.0%	97.5%	97.5%
		Absorbance at 505 nm				
50°C for 20 min	0	0.000	0.001	0.003	0.003	0.003
	40	0.238	0.240	0.242	0.237	0.235
	60	0.358	0.357	0.356	0.353	0.351
	100	0.589	0.582	0.580	0.577	0.570
		(Absorbance/0 time delay absorbance) × 100				
	40		100.5%	101.4%	99.6%	98.7%
	60		99.9%	99.4%	98.7%	98.0%
	100		98.8%	98.6%	98.0%	96.9%
		Time delay to reading, min				
		0	10	20	30	40
Standard solution:GOPODk reagent (0.1:3.0)		Absorbance at 505 nm				
50°C for 20 min	0	0.000	0.002	0.004	0.000	0.001
	400	0.455	0.453	0.452	0.445	0.444
	600	0.674	0.671	0.671	0.662	0.656
	1000	1.114	1.107	1.098	1.087	1.079
		(Absorbance/0 time delay absorbance) × 100				
	400		99.6%	99.3%	97.8%	97.5%
	600		99.5%	99.5%	98.1%	97.3%
	1000		99.4%	98.6%	97.6%	96.9%

[a] Values presented as least-squares means and calculated percentages.

ratio permutation. The comparisons that were tested were selected based on the outcomes of preceding experiments, with focus on achieving the study goals of reducing the time required for the assay, achieving accurate predictions of glucose concentrations, and evaluating factors that affect the outcome of the modified assays.

Data within each experiment were analyzed as a completely randomized design with method, glucose concentration of the standard solution, and the sample by method interaction included in the statistical model. Numeric factors, such as time to analysis of a solution, were treated as continuous variables to determine statistical significance and classification variables to calculate the least-squares means. If an interaction term was not significant, a reduced model with the interaction term removed was analyzed to determine the significance of the main effects. When appropriate, batches of GOPOD reagent used or assay run were included as random variables. Statistical analysis was performed using the Mixed procedure of the SAS software (SAS Version 8, SAS Institute, Cary, NC). Standard curve equations, residual plots, R^2, root mean squared error, and sum of squared residuals (residual = observed minus predicted value) were determined using the Reg (regression) procedure of SAS.

The effect of the number of glucose standard solutions used on the accuracy of the prediction of quadratic standard curves calculated from the standards was tested. Concentrations of glucose in the standard solutions were predicted using the standard curves and the measured absorbances of the standards. Accuracy of prediction was evaluated using the residuals as the response variable (actual glucose concentration minus predicted glucose concentration). The statistical model included number of glucose standards (3, 4, or 5) used for calculation of the curve within the day in which the analysis was performed, glucose concentration of the standard solutions, and the interaction of these terms. All factors were used as classification variables.

Materials

Purified D-glucose (>99.5% purity; Sigma-Aldrich, Inc., St. Louis, MO; used as purchased) was used to prepare the standard solutions. Average dry matter content of glucose was determined with drying for 15 h at 105°C in a forced-air oven. Glucose values were adjusted for dry matter and purity (determined by manufacturer; 99.8–99.9%).

Apparatus

A spectrophotometer capable of operating at absorbances of 505 and 510 nm was used (Ultrospec 3000 UV-Vis spectrophotometer, Pharmacia Biotech, Model 80-2106-20, Cambridge, UK).

Reagents and Solutions

All reagents and solvents were analytical reagent grade. All references to water are for distilled or equivalent reverse osmosis purified water.

(a) *Glucose oxidase–peroxidase–aminoantipyrine buffer mixture.—(1) GOPODk (for Karkalas method).*—Mixture of glucose oxidase, 7000 U/L; peroxidase, 7000 U/L; and 4-aminoantipyrine (also called 4-aminophenazone, $C_{11}H_{13}N_3O$, CAS 83-07-8; not to be confused with 4-*N*,*N*-dimethyl aminophenazone, also known as aminophenazone or aminopyrine), 0.74 mM in a buffer. Prepared by dissolving 9.1 g Na_2HPO_4 and 5.0 g KH_2PO_4 in ca 300 mL H_2O in a 1 L volumetric flask. Used H_2O to rinse chemicals into bulb of flask. Swirled to dissolve completely. Added 1.0 g phenol and 0.15 g 4-aminoantipyrine. Used H_2O to rinse chemicals into bulb of flask. Swirled to dissolve completely. Added glucose oxidase (7000 U) and peroxidase (7000 U), rinsed enzymes into flask with H_2O, swirled gently to dissolve without causing excessive foaming. Diluted to 1 L with H_2O. Sealed. Inverted repeatedly to mix. Filtered solution through a glass fiber filter with 1.6 μm retention and stored in a sealed amber bottle at ca 4°C. Reagent should be used within 1 month.

*(2) GOPODa (for GOPOD assay in AOAC Method **996.11**).*—Mixture of glucose oxidase, 12 000 U/L; peroxidase, 650 U/L; and 4-aminoantipyrine, 0.4 mM in a buffer containing KH_2PO4, NaOH, and 4-hydroxybenzoic acid adjusted to pH 7.4. Buffer was prepared by dissolving 13.6 g KH_2PO_4, 4.2 g NaOH, and 3.0 g 4-hydroxybenzoic acid in 96 mL H_2O. pH was adjusted to 7.4 with either 2 M HCl or 2 M NaOH and solution diluted to 100 mL; sodium azide was not added. The entire buffer mix was transferred to 2 L volumetric flask and the solution made to contain 0.4 mM 4-aminoantipyrine, >12 000 U/L of glucose oxidase, and >650 U/L peroxidase. The solution was gently swirled to dissolve enzymes and chemicals, and was diluted to volume. The reagent was filtered through a glass fiber filter with 1.6 μm retention and stored in a sealed bottle at ca 4°C. Reagent is stable for 2 to 3 months at 4°C.

Note: Glucose oxidase (Sigma-Aldrich product G-6125) contained 21 200 U glucose oxidase/g solid and 0.0461 U catalase/mg solid (manufacturer's analysis).

(b) *Glucose standard solutions.*—Standard solutions were made independently, with glucose weighed separately for each solution in order to avoid the issue of improper preparation of a stock solution or pipetting issues affecting the accuracy of the standard solutions. For all standard solutions, glucose was weighed on an analytical balance, and weight was recorded to 0.0001 g; the glucose was quantitatively transferred with rinsing to a volumetric flask, dissolved, and diluted to volume. Dry matter of powdered crystalline glucose (purity ≥99.5%) was determined by drying for 15 h at 105°C in a forced-air oven. The weight of glucose added to a flask was multiplied by dry matter percentage and assayed purity of the glucose (provided by manufacturer) and divided by dilution volume milliliter to calculate actual glucose concentrations of the solutions. For glucose concentrations between 0 and 100 μg/mL, the amount of glucose weighed ranged from 0 to 50 mg and dilution volume was 500 mL; for concentrations between 0 and 1000 μg/mL, the glucose amount ranged from 0 to 250 mg and dilution volume was 250 mL. For samples prepared in benzoic acid solution, 0.2% benzoic acid (w/v) solution was substituted for water.

Table 2. Historic data and results of the present study on effect of standard solution preparation on form of standard regression curve

	Curve form[a]	Intercept	Coefficient for abs[b]	Coefficient for abs squared	R^2	RMSE[c]	Quadratic term *P*-value	Sum of squared residuals
Trinder, 1969 (ref. 8)	L	–5.782	505.536		0.9996	7.03		197.5
	Q	0.559	472.30	16.36	1.0000	1.48	<0.01	6.56
Time, min[d]			Fresh glucose solutions in water, 7 point standard curves					
Standard solution:GOPODk reagent[e] (0.1:3.0)								
45	L	–4.267	899.68		0.9997	4.743		890.0
45	Q	0.342	871.47	25.9859	0.9999	3.402	<0.01	451.3
140	L	–1.794	899.60		0.9997	5.067		1027.0
140	Q	3.338	867.66	29.6173	0.9999	3.342	<0.01	435.6
380	L	–0.318	899.60		0.9998	4.502		810.8
380	Q	3.028	878.92	19.1439	0.9998	3.802	<0.01	563.8
Standard solution:GOPODk reagent (0.5:2.5)								
45	L	–0.277	169.78		0.9999	0.368		5.41
45	Q	–0.007	166.74	5.1439	0.9999	0.321	<0.01	4.03
140	L	–0.205	169.13		0.9997	0.532		11.32
140	Q	–0.001	166.83	3.8836	0.9997	0.520	0.03	10.56
380	L	–0.180	169.99		0.9999	0.369		5.45
380	Q	0.012	167.82	3.6773	0.9999	0.349	0.01	4.76
Time, days			Benzoic acid solutions, 5 point standard curves					
Standard solution:GOPODk reagent (0.1:3.0)								
1	L	–4.054	903.22		0.9998	4.851		305.9
1	Q	0.007	873.80	26.6310	0.9999	3.307	<0.01	131.2
2	L	–5.232	900.70		0.9998	5.183		349.2
2	Q	0.137	861.88	35.0511	1.0000	1.972	<0.01	46.7
3	L	–4.669	900.55		0.9999	3.964		204.2
3	Q	–0.405	869.83	27.6968	1.0000	1.061	<0.01	13.5
Standard solution:GOPODk reagent (0.5:2.5)								
1	L	–0.210	170.87		0.9999	0.442		2.54
1	Q	0.112	166.43	7.6165	0.9999	0.346	0.01	1.44
2	L	–0.064	170.02		0.9999	0.318		1.32
2	Q	0.126	167.40	4.4834	0.9999	0.278	0.04	0.93
3	L	–0.468	170.28		0.9998	0.488		3.10
3	Q	–0.007	163.97	10.7596	0.9999	0.269	<0.01	0.87

[a] Curves represent combined results of 2 replicate assay runs. L = Linear, Q = quadratic.
[b] Abs = Absorbance.
[c] RMSE = Root mean squared error.
[d] Time from standard solution preparation to analysis and reading of absorbance of samples.
[e] GOPODk = Glucose oxidase–peroxidase reagent of Karkalas (ref. 5).

Procedures

In the general procedure used for each assay run, the specified volumes of H_2O and glucose standard solutions were pipetted in triplicate into the bottom of 16 mm diameter glass culture tubes (100 or 150 mm height) to give 3 tubes per standard per treatment. The specified volume of GOPOD was added to each tube using a positive displacement repeating pipet. If tubes were vortexed, it was done at this point. Tubes were covered with plastic film and incubated at the specified temperature and time in a water bath capable of maintaining the temperature ±1°C. Post-incubation cooling was performed after removal from the water bath. The spectrophotometer was zeroed to water, and sample absorbance was read at 505 nm for GOPODk and 510 nm for GOPODa. Absorbance values corrected for the average of the 0 μg glucose/mL solutions for each treatment were calculated and used in calculation of standard curves. Equations for linear and quadratic forms of glucose standard curves were calculated where Y = glucose μg/mL and X = absorbance to reflect the form of the equation used to predict glucose concentrations of unknown samples. Glucose amounts per tube used in this assay (0–100 μg) were within the range in which the original protocol indicated that Beer's law was obeyed (5).

The variations of the GOPODk procedure evaluated were:

(**a**) *Effect of incubation conditions.*—0.5 mL volumes of 0, 40, 60, and 100 μg/mL glucose standard solutions with 2.5 mL GOPODk reagent were mixed on a Vortex mixer, incubated at 35°C for 45 min or 50°C for 20 min, and cooled for 10 min in the dark before having their absorbance read immediately at 505 nm; or, after samples were held on the bench, they were read at 15, 30, 45, and 60 min thereafter. The 35°C for 45 min incubation represents the original method (5). An incubation temperature of 60°C for 20 min was also evaluated, but not pursued because measured absorbances were 13% lower than those obtained at 50°C for 20 min incubations (P <0.01 for effect of temperature; data not shown).

(**b**) *Effect of post-incubation cooling in dark.*—Standard solutions with GOPODk were prepared as in (**a**) for 20 min at 50°C incubation, except that absorbances were read immediately after the incubation or after samples were cooled for 10 min in the dark.

(**c**) *Alteration of the ratio of standard solution:GOPODk reagent volume.*—0.1 mL volumes of 0, 400, 600, and 1000 μg/mL glucose standard solutions with 3.0 mL GOPODk were mixed on a Vortex mixer, incubated at 50°C for 20 min, and cooled for 10 min in the dark before having their absorbance read at 505 nm. Effect of inclusion or omission of the 10 min post-incubation cooling in the dark, and effect of reading absorbances immediately after incubation, or at 10, 20, 30, and 40 min thereafter were evaluated.

(**d**) *Effect of inclusion or omission of vortexing step.*—The effect of inclusion or omission of the step in which standard solution with GOPODk was mixed on a Vortex mixer before incubation was evaluated.

(**e**) *The effect of time delay.*—The effect of time delay from preparation of glucose standard solutions to the time they were analyzed and read on the spectrophotometer was evaluated to indirectly assess the effect of mutarotation of glucose on the form of the standard curves (glucose oxidase is specific for the β-anomer of glucose). Glucose solutions prepared fresh daily in H_2O and glucose in 0.2% w/v benzoic acid solution were used. Freshly prepared solutions contained ca 0, 20, 40, 50, 60, 80, and 100 μg glucose/mL for the standard solution:GOPOD (0.5:2.5) ratio, and 0, 400, 500, 600, 700, 800, and 1000 μg glucose/mL for the standard solution:GOPOD (0.1:3.0) reagent ratio. Benzoic acid solutions contained ca 0, 25, 50, 75, and 100 μg glucose/mL for the standard solution:GOPOD (0.5:2.5) ratio, and ca 0, 250, 500, 750, and 1000 μg glucose/mL for the standard solution:GOPOD (0.1:3.0) ratio. Time from preparation of the solutions to reading absorbance at the end of the glucose assay were approximately 45, 140, and 380 min for freshly prepared solutions, and 1, 2, and 3 days for benzoic acid solutions. All solutions were held at ambient temperature until analysis.

(**f**) *Alternative GOPOD glucose assay.*—An alternative GOPOD glucose assay method (3) using a different GOPOD reagent was evaluated to determine whether the quadratic form of the standard curve was found only in the Karkalas method with GOPODk. The AOAC GOPODa formulation and incubation conditions described in AOAC Method **996.11** (3) were used with glucose solutions prepared with 0.2% benzoic acid solution as described in (**e**) 9 days after the glucose standards were prepared. The GOPODk reagent was also used to analyze the same glucose solutions on the same day for comparison of absorbance per μg glucose/mL.

(**g**) *Effects of form of the standard curve.*—Effects of form of the standard curve and number of standards included in the curve on predictions of starch content of samples were estimated mathematically. Linear and quadratic curves prepared with 5 standard solutions were evaluated, as well as a linear standard curve using only the 0 μg glucose/mL and greatest-concentration standard solutions. Data from the analysis performed using GOPODk and glucose standards prepared in 0.2% benzoic acid 3 days before the assay was performed were used to generate the standard curves. To calculate the effects of deviations in the glucose predictions on sample starch concentrations, the average measured absorbance of each standard solution was entered into the standard curve regression equations to calculate predicted glucose concentrations of the standards. The actual glucose concentrations were subtracted from the predicted values to give μg glucose/mL values for the deviation of the predicted vs actual glucose concentration for each standard. The predicted concentration minus actual μg glucose/mL values were multiplied by 0.9 to convert glucose to a starch basis, then multiplied by a dilution factor (1000 or 100 for 0.5:2.5 and 0.1:3.0 sample solution:GOPODk, respectively), then divided by 1 000 000 to convert from micrograms to grams, and finally divided by 0.09 to represent a 0.1 g sample with a dry matter content of 90%. The calculated value was multiplied by 100 to covert to a percentage basis.

Table 3. Standard curves for AOAC glucose detection method[a]

Run	Curve form[b]	Intercept	Coefficient for abs[c]	Coefficient for abs squared	R^2	RMSE[d]	Quadratic term *P*-value	Sum of squared residuals
			Standard solution:GOPODa reagent[e] (0.1:3.0)					
A	L	–4.030	947.27		0.9999	4.084		216.9
A	Q	–0.190	918.14	27.6426	1.0000	2.261	<0.01	61.3
B	L	–7.374	949.99		0.9997	6.465		543.4
B	Q	–0.970	901.44	46.1339	0.9999	3.029	<0.01	110.1
			Standard solution:GOPODa reagent (0.5:2.5)					
A	L	–0.438	179.99		0.9999	0.419		2.29
A	Q	–0.041	174.28	10.2969	1.0000	0.232	<0.01	0.64
B	L	–0.576	178.95		0.9995	0.777		7.85
B	Q	0.071	169.61	16.7836	0.9998	0.531	<0.01	3.39

[a] Curves represent individual assay runs.
[b] L = Linear, Q = quadratic.
[c] Abs = Absorbance.
[d] RMSE = Root mean squared error.
[e] GOPODa = Glucose oxidase–peroxidase reagent (ref. 3).

The effect of the number of glucose standards used to calculate the quadratic standard curve on the accuracy of predicted values was tested using data from (**e**) for glucose standards 2, 3, and 9 days after they were prepared (3 analysis runs, 1 per day). Standard curves were calculated for each separate run with data from 3 (highest, lowest, and midpoint), 4 (2 lowest, 2 highest), and 5 (all standards) glucose concentrations. The standard curves were used to calculate predicted values for the glucose concentrations of all 5 standard solutions, and the actual minus predicted residual values for glucose concentrations of the solutions were calculated.

(**h**) *Effect of a hydrophilic antioxidant (ascorbic acid) on glucose detection.*—Ascorbic acid was dosed in μmol quantities reported for hydrophilic antioxidants in foodstuffs (6) into glucose samples carried through a starch assay procedure. A solution of 5000 μmol ascorbic acid/L was prepared with 0.1 M sodium acetate buffer (pH 5.0) used as the diluent. The ascorbic acid solution was pipetted into both reagent blank tubes and tubes containing 100 ± 0.2 mg glucose before addition of 0.1 M sodium acetate buffer (pH 5.0) to achieve a total volume of 30 mL. Ascorbic acid solution was added to provide 0, 1, 2.5, 5, 10, 20, 30, or 50 μmol ascorbic acid per tube. Single treatment tubes for each substrate and ascorbic acid addition were analyzed in each of 2 runs. Sample solutions were analyzed in triplicate using 2 ratios of sample solution:GOPODk (0.1:3.0 and 0.5:2.5) incubated for 20 min at 50°C.

Starch Analysis Method

A modification of the method of Bach Knudsen (7) was performed on D-glucose with ascorbic acid solution additions. Purified D-glucose was accurately weighed into 25 × 150 mm screw-cap glass tubes. The desired volume of 5000 μmol ascorbic acid/L was dispensed into tubes containing glucose or no substrate (reagent blanks). Sodium acetate buffer (0.1 M, pH 5.0) was added to each tube to bring the liquid volume to 30 mL. Heat-stable α-amylase (0.1 mL, ca 2000 Liquefon units; Spezyme Fred, Genencor International, Inc., Rochester, NY; origin: *Bacillus licheniformis*; "Liquefon unit" is a measure of α-amylase activity for which a detailed assay is available from the manufacturer) was pipetted into each tube, which was then capped and mixed on a Vortex mixer. Tubes were incubated for 1 h at 100°C, with vortexing at 10, 30, and 50 min of incubation. After cooling on the bench for 0.5 h, 1 mL amyloglucosidase solution (200 U/mL in 0.1 M sodium acetate buffer, pH 5.0) was added, tubes were mixed on a Vortex mixer, then incubated for 2 h at 60°C, with vortexing at 1 h. After incubation, 20 mL H_2O was dispensed into each tube, and the tubes were recapped and inverted to mix. From each tube 1.5 mL of solution was transferred to a 2 mL microcentrifuge tube, then centrifuged at 1000 × *g* for 10 min. The centrifuged solutions were allowed to come to room temperature before preparing them in 1:1 dilutions with H_2O for glucose analysis.

Results and Discussion

Incubation Conditions

No effect of incubation time and temperature was detected for 0.5 mL of standard solution with 2.5 mL of GOPODk reagent incubated at 35°C for 45 min or 50°C for 20 min, and held 10 min in the dark at ambient temperature before reading (P = 0.79). Nor was there an interaction of incubation conditions and glucose concentration of the standard solutions

Table 4. Absorbance per μg glucose/mL standard solution[a]

	Sample solution:GOPOD reagent				
	0.1:3.0			0.5:2.5	
	Karkalas[b]	AOAC[b]		Karkalas	AOAC
	Glucose μg/mL				
250	0.00116	0.00110	25	0.00617	0.00574
500	0.00113	0.00108	50	0.00604	0.00568
750	0.00113	0.00106	75	0.00598	0.00563
1000	0.00112	0.00106	100	0.00592	0.00557
S_r					
P-value of quadratic term	0.05	0.03		0.02	0.98

[a] Values are least-squares means.

[b] Karkalas: GOPODk used (ref. 5); AOAC: GOPODa used (ref. 3).

($P = 0.47$), indicating that the standard curves did not diverge over the range of 0–100 μg glucose/mL concentrations. The respective standard curves were glucose μg/mL = $169.25x - 0.16$ at 35°C for 45 min (adjusted $R^2 = 0.9997$), and $169.78x - 0.17$ at 50°C for 20 min (adjusted $R^2 = 0.9998$), where x = the measured absorbance.

Delayed reading after incubation altered the absorbance of standard solutions ($P < 0.01$): those containing glucose declined, but the 0 μg/mL standard increased slightly over time (Table 1). The absolute decrease in absorbance with time was greater for greater concentrations of glucose ($P < 0.01$). Incubation conditions ($P = 0.66$) and the interaction of incubation conditions and glucose concentration ($P = 0.16$) did not alter the effect of delayed time to reading. Absorbance declined at approximately 0.5% each 15 min. The recommendation in the original protocol (5) that samples be read before 30 min would allow a decrease to ca 99% of the initial absorbance. With comparable responses in absorbance and delay to reading, the shorter incubation time at warmer temperature could be used to give results equivalent to those of the original method.

Allowing samples time to cool in the dark between incubation and absorbance reading did not affect results for the standard solution:GOPODk (0.5:2.5) samples incubated at 50°C for 20 min. Neither reading samples immediately after removal from the water bath, nor cooling the samples in the dark (least-squares means for absorbances 0.299 and 0.298, respectively; $P = 0.92$), nor the interaction of cooling in the dark by glucose concentration ($P = 0.62$) affected sample absorbance. Mixing the sample solution with GOPODk on a Vortex mixer before incubation also not did not affect sample absorbance for the 20 min at 50°C incubated samples (least-squares means for absorbances: 0.296 vortexed, 0.296 not vortexed; $P = 0.89$; glucose concentration × vortex, $P = 0.54$).

Ratio of Standard Solution:GOPODk Reagent (0.1:3.0)

Changing the ratio of standard solution:GOPODk reagent from the 0.5:2.5 described in the original protocol to 0.1:3.0 incubated at 50°C for 20 min with glucose concentrations of 0–1000 μg/mL increased the amount of glucose added per reaction tube, but was still within the range reported to obey Beer's law in the original protocol (5). By using a smaller sample volume and a broader range of glucose concentrations for the standard curve, the need for or extent of sample dilution is reduced (3). The standard curves produced with this modification had much greater slopes and intercepts (e.g., glucose μg/mL = $896.24x - 3.39$; $R^2 = 0.9999$); x = measured absorbance.

Absorbance values were reduced for samples that were cooled in the dark compared to those read immediately, with the difference increasing with increasing glucose concentration ($P < 0.01$; for μg glucose/mL of 0, 399, 600, and 999, absorbance at 505 nm: 0.019, 0.471, 0.694, and 1.130 for samples read immediately, and 0.020, 0.469, 0.690, and 1.120 for those cooled in the dark for 10 min, respectively; values are least-squares means; interaction of post-incubation cooling in the dark by glucose concentration, $P < 0.01$). Unlike the 0.5:2.5 ratio of standard solution:GOPODk, for which results were not affected, use of a cooling period is not recommended for the 0.1:3.0 ratio.

As with the 0.5:2.5 ratio of standard solution:GOPODk with delayed reading of samples, the absorbance of the 0 μg/mL solutions increased slightly over time, while the glucose solutions declined at a rate of approximately 1% every 20 min (Table 1). Vortexing the sample solution with GOPODk reagents before incubation not did not affect sample absorbance (vortex, $P = 0.78$; glucose concentration × vortex, $P = 0.94$).

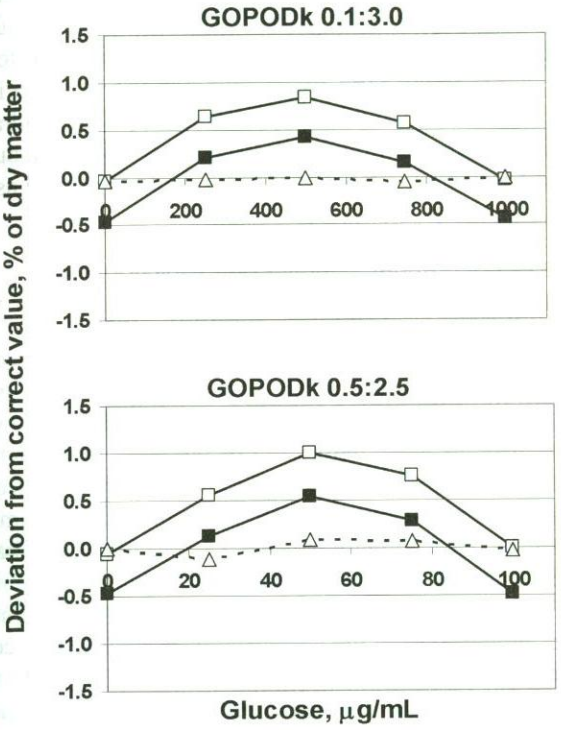

Figure 1. Effect of deviations in predicted glucose concentrations on calculated estimates of sample starch content as related to form of standard curve used. GOPODk = GOPOD reagent of (ref. 5), 0.1:3.0 and 0.5:2.5 are the ratios of standard solution:GOPOD used. ■ = 5-point linear equation, □ = 2-point linear equation, and △ = 5-point quadratic equation.

Linearity of Response

A problematic aspect of the regression equations produced from all approaches used with GOPODk was that all the linear equations had R^2 of nearly 1.0 (0.9998 to 1.0), suggesting a very good fit to the linear form, but the intercept was not 0. Thus, when the standard curves were used to predict glucose concentrations of the standard solutions used to produce them, the predicted values were frequently incorrect. It was determined that a quadratic form fit the standard curves better than a linear form (Table 2), based on significance of the quadratic term in the regression equation, the reduction in the root mean squared error of the standard curve, and the relative decrease in residual sums of squares (residual = observed minus predicted) between the linear and quadratic equations, and evaluation of the residual vs predicted value plots. Other nonlinear forms were not explored. Review of data from one of the original GOPOD assays for glucose (8; Table 2), as well as of glucose assays performed with the original GOPODk method (5) at 3 different institutions with different equipment over the course of 13 years frequently showed the non-zero intercept and quadratic pattern of the standard curve (data not shown). Presence of catalase in the glucose oxidase enzyme did not seem to be implicated as the ratio of peroxidase to catalase in the GOPODk reagent in the present study was 460:1. Catalase has a considerably lower Km for H_2O_2 than does peroxidase (93 and <5 mM, respectively; 9) and the maximal millimolar concentration of glucose in the standard solution + GOPODk reaction mixes was 32.3.

In the original work (5), absorbance of glucose solutions was measured against a 0 μg glucose/mL solution to which GOPODk had been added, though the author did not indicate whether the 0 standard was included in the standard curve. Even with exclusion of the 0 μg glucose/mL absorbances from calculation of the standard curves in the present data set, the quadratic term remained significant, and the pattern of residuals for the linear form of the curve still suggested that the curves were not linear (data not shown). The quadratic/nonlinear form of the curve does not appear to be due to inclusion of a 0 standard.

Investigations into the need for equilibration of α- and β-anomers of glucose in the standard solution, as evaluated by allowing different periods of time to elapse between preparation of the glucose solutions and their analysis, and effects of different GOPOD reagents suggest that the nonlinear/quadratic absorbance response to glucose is inherent in this assay. Both for the standard solutions prepared fresh daily and those made in benzoic acid solution, and for the different ratios of standard solution to GOPODk reagent, the quadratic terms of the curves were significant, and the values for the sum of squared residuals and root mean square error were smaller for the quadratic than for the linear forms of the equations (Table 2).

Specific to the standards prepared fresh daily with H_2O, time from preparation of the standards to reading of samples did not affect absorbances for the 0.1:3.0 ratio of standard solution:GOPODk reagent (time × glucose concentration, $P = 0.96$; reduced model time, $P = 0.15$; least-squares means for absorbance: 0.639, 0.636, and 0.634, for 45, 140, and 380 min, respectively; standard error of the difference = 0.0031; Table 2). For the ratio of standard solution:GOPODk (0.5:2.5), the interaction of glucose concentration and time was not significant ($P = 0.35$), but time did affect absorbance ($P < 0.01$ in the reduced model; least-squares means for absorbance: 0.298, 0.296, and 0.295, for 45, 140, and 380 min, respectively; standard error of the difference = 0.0007). This result is in contrast to results in the original protocol in which α- and β-anomers were reported to have equilibrated by 40 min into the 35°C incubation (5). The quadratic terms of all standard curves were significant.

For glucose standards prepared in 0.2% benzoic acid solution, the time between preparation of the standards and their analysis and reading did not affect the standard solution:GOPODk (0.1:3.0) samples (time by glucose concentration, $P = 0.53$, reduced model time $P = 0.22$; least-squares means for absorbance: 0.557, 0.560, and 0.560 for 1, 2, and 3 days after standard preparation, standard error of the difference: 0.0009; Table 2). For the sample solution:GOPODk (0.5:2.5) samples, the time to analysis did

Table 5. Effect of number of glucose standards used for calculation of quadratic standard curves on accuracy of prediction of glucose concentrations in the standards[a]

		Actual minus predicted glucose, μg/mL	
		Sample solution:GOPODk reagent	
Day of analysis	No. of glucose standards	0.1:3.0	0.5:2.5
2	3	–0.243	0.064
2	4	0.161	–0.044
2	5	<0.001	<0.001
3	3	0.170	0.047
3	4	–0.113	–0.032
3	5	<0.001	<0.001
9	3	–0.615	–0.46
9	4	0.542	0.032
9	5	0.017	<0.001
Standard error of the mean		0.456	0.062

[a] Values are least-squares means for each standard curve.

affect absorbances (time by glucose concentration, $P = 0.44$; reduced model time, $P < 0.01$; least-squares means: 0.293, 0.294, and 0.296 for 1, 2, and 3 days after standard preparation; standard error of the difference: 0.0005). Overall, the standard solution:GOPODk (0.1:3.0) standards appeared to be less affected by time of standard preparation than were the 0.5:2.5 samples.

The use of the GOPODa reagent that used more units of glucose oxidase, used fewer units of peroxidase, and used 4-hydroxybenzoic acid rather than phenol gave similar results to the GOPODk assay (Table 3). All standard curves produced with GOPODa were more quadratic than linear, as determined on the basis of significance of the quadratic term, reduction in root mean squared error, and sum of squared residuals between the linear and quadratic forms of the curves.

Although the original assay reported a linear response in absorbance through 200 μg glucose/mL (5), the nonlinear nature of the relationship of the absorbance per unit of glucose and non-zero intercept of the linear equations indicate that this is perhaps not the best model (Table 4). In agreement with the original study, the relationship between absorbance and glucose concentration in the present study became grossly nonlinear and in violation of Beer's Law (absorbance response plateaued or declined with increasing glucose concentrations) at approximately 300 and 1500 μg glucose/mL for the ratios of sample solution:GOPODk (0.5:2.5 and 0.1:3.0), respectively (data not shown). The original basis for presuming linearity of the responses at glucose concentrations <200 μg glucose/mL probably lies in the very high R^2 for the linear form of the curves, and in that the absorbance per unit of glucose values differ in the fourth or fifth decimal place. While the quadratic form seems to fit better than the linear form, we do not necessarily consider it to be the "true" or "best" form of the relationship. The quadratic form is presented as a clear improvement over linearity, but it is possible that other functional forms could fit as well as or better than the quadratic.

Impact of Standard Curves on Prediction and Implications

Linear or not, the value of an assay is in its ability to predict with the desired accuracy the content of an analyte in a substrate. Both the GOPODk and GOPODa methods showed similar patterns when the impact of predicted minus actual glucose concentrations of standard solutions was calculated to apply to determination of the starch content of a 0.1 g sample of 90% dry matter (Figure 1; GOPODk data only). Quadratic curves produced from 5 glucose standards showed no more than 0.1% deviation from the correct value, whereas linear curves produced from the same data over-predicted glucose concentration and calculated starch content through the middle of the range of standard solutions by up to 0.5% of sample dry matter, and under-predicted by the same amount at the highest and lowest concentrations. The linear curve produced from the highest and zero glucose standards gave accurate predictions at these 2 points, but overestimated in the middle of the standard curve by up to 1% of dry matter. The different standard solution:GOPOD ratios behaved similarly when 100× and 1000× dilution factors were used for the 0.1:3.0 and 0.5:2.5 ratios, respectively. These dilution factors allow samples containing 0.09 g of pure starch (e.g., pure starch with a dry matter of 90%) to fall into the range of the standard curve. Greater dilution of such samples may allow them to be read in the middle of the standard curve; however, increasing the dilution factor also multiplies the size of the error [e.g., compared to 1000×, a 2000× dilution factor would double the overestimation midrange on the 5-point linear curve for the sample solution:GOPOD (0.5:2.5) ratio]. Use of greater sample size while staying within the 0.09 g of starch limit can also reduce error as the greater sample weight is divided into the starch estimate (e.g., a 0.2 g sample would have half the predicted minus actual deviation of a 0.1 g sample). The error will vary somewhat depending upon the standard curve run.

Depending on the desired accuracy, linear or quadratic standard curves can be used, but the quadratic equation gives more accurate predictions. With possible deviations of –1 to +1, or 0 to +2 percentage units from the accurate value depending on how the standard curve is run, dilution factor, and where in the standard curve the sample absorbances fall, interpretation of single measures, such as clinical blood glucose values, would be little affected whether linear or quadratic equations are used. However, such deviations could skew interpretation of results or mask differences when values are used for comparison, such as for starch contents among grain varieties or efficiency of yield of ethanol from starch in

Table 6. Effect of ascorbic acid additions on absorbance of glucose samples carried through a starch analysis[a]

	Sample solution:GOPODk reagent, mL:mL			
	0.1:3.0		0.5:2.5	
Ascorbic acid, μmol	Absorbance, 505 nm[b]	0 μmol ascorbic acid absorbance, %	Absorbance, 505 nm[c]	0 μmol ascorbic acid absorbance, %
0	1.080	100.0	0.566	100.0
1	1.084	100.4	0.567	100.2
2.5	1.079	99.9	0.569	100.5
5	1.080	100.0	0.566	100.0
10	1.072	99.3	0.565	99.8
20	1.047	97.0	0.559	98.7
30	1.036	96.0	0.549	97.0
50	0.982	91.0	0.534	94.3

[a] Values are least-squares means.
[b] Standard error of the difference for least-squares means = 0.0049.
[c] Standard error of the difference for least-squares means = 0.0017.

batches of corn grain. Another way that use of linear GOPOD standard curves may affect accuracy is by compensating for or adding to other errors in assays in which the GOPOD method is incorporated. For example, in starch assays in which samples are gelatinized and hydrolyzed with heat-stable α-amylase at neutral pH, maltulose formation should decrease recovery of starch as released glucose (10). However, overestimation of glucose in the middle of the linear standard curves may provide a compensating error, allowing values for purified starches to measure closer to 100%.

Evaluation of the effect of the number of glucose standard solutions used to generate a quadratic standard curve within a given run showed that use of standard curves produced using 3, 4, or 5 standard solutions did not differ in their accuracy of prediction [$P > 0.80$ for ratios of sample solution:GOPODk (0.1:3.0 and 0.5:2.5); Table 5]. Neither the effect of glucose concentration ($P > 0.58$) nor the interaction of glucose concentration and number of standards used to generate the curves ($P > 0.87$) were significant for either sample solution:reagent ratio. The curves generated from 5 glucose standards had the numerically smallest residuals, but even the largest residual [–0.615 μg glucose/mL for sample solution:reagent (0.1:3.0)] was small. Even though the accuracy was acceptable, we do not recommend using 3 glucose concentrations to describe a quadratic curve, as this is overfitting the data and risks generation of an erroneous curve if one of the glucose standards is not properly prepared. Use of 4 glucose standards to produce standard curves gives acceptable results.

Repeatability

Repeatability of absorbance values on triplicate samples of standard solutions was very good within run and is a key reason that the small deviations from linearity could be detected. For the glucose standards prepared in benzoic acid, the standard solution:GOPODk ratio (0.1:3.0) gave standard errors of 0.0022, 0.0012, 0.0018, 0.0021, and 0.0030 for 0, 250, 500, 750, and 1000 μg glucose/mL standards (overall coefficient of variation % for glucose-containing solutions = 0.31%). Standard error values for the 0.5:2.5 ratio were 0.0011, 0.0016, 0.0018, 0.0013, and 0.0016 for 0, 25, 50, 75, and 100 μg glucose/mL standards (overall coefficient of variation % for glucose-containing solutions = 0.57%). Values for the freshly prepared samples were similar.

Limit of Determination

Limits of determination for glucose measurement were calculated from absorbance values of 0 μg glucose/mL standards analyzed in triplicate from 4 assay runs in which the glucose standards were prepared in 0.2% benzoic acid solution. Values were calculated as mean blank value + 3 × blank standard deviations (11). For standard solution:GOPODk (0.5:2.5 and 0.1:3.0) samples, the mean absorbance ± standard deviation of undiluted blanks were 0.0002 ± 0.0010 for a detection limit of 0.0029 absorbance, and 0.0002 ± 0.0020 for a detection limit of 0.0063 absorbance, respectively. Using average quadratic standard curves calculated for each preparation, glucose detection limits are 0.53 and 5.12 μg/mL for standard solution:GOPODk (0.5:2.5 and 0.1:3.0), respectively. The detection limits represent approximately 0.5% of the range of the glucose standards in each case.

Effect of Antioxidants

Addition of ascorbic acid to tubes containing glucose and subject to a modification of the Bach Knudsen (7) assay for starch showed a linear decrease in absorbance at additions of >10 μmol of ascorbic acid [effect of ascorbic acid on absorbance for 0–10 μmol of ascorbic acid, $P = 0.30$ and 0.37 for sample:GOPODk (0.1:3.0 and 0.5:2.5), respectively;

effect of ascorbic acid for 10–50 μmol: linear $P < 0.01$ and quadratic $P < 0.01$ for 0.1:3.0 and 0.5:2.5 sample:GOPODk, respectively; Table 6]. The effect was relatively small through 20 μmol ascorbic acid. Investigations into the antioxidant content of foodstuffs (6) showed that most of the high starch or leafy vegetable foods had hydrophilic antioxidant values that would be equivalent to <10 μmol of ascorbic acid per 0.1 g dry matter. Exceptions included foods high in phenolic compounds (e.g., beets, red sorghum grain, antioxidant content approximately equivalent to 23 and 14 μmol ascorbic acid, respectively). Because of the interference in the GOPOD assay, another method for measuring glucose should be considered for feeds or foods exceeding 10–20 μmol of hydrophilic antioxidant per 0.1 g dry matter.

Recommendations

Based on its lesser sensitivity to time of sample preparation, the ratio of standard solution:GOPODk reagent (0.1:3.0) incubated at 50°C for 20 min is the preferred approach among those tested. With the reduced incubation time, and no need to mix samples on a Vortex mixer or cool them in the dark after incubation, a reduction in 30–40% of the time needed to perform the assay can be realized. Absorbance of samples should be read within 30 min of incubation. Use of a quadratic form of the standard curve produced using a minimum of 4 standard solutions differing in glucose concentration will give greater accuracy of prediction as compared to linear equations, but the choice in form of the equation depends on the accuracy required for the application. Use of standard solutions prepared in advance in 0.2% benzoic acid solution reduces the time needed to run the assay and avoids potential issues with solubilization or equilibration of glucose. This assay may be used to analyze materials with ≤10 μmol of hydrophilic antioxidant per 0.1 g of air-dried sample without appreciable reduction in glucose values, and reductions are small through 20 μmol, but an alternative glucose assay should be considered for use on samples containing more antioxidant.

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